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Hypoxanthine Incorporation Is Nonmutagenic in *Escherichia coli*

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Endonuclease V, encoded by the *nfi* gene, initiates removal of the base analogs hypoxanthine and xanthine from DNA, acting to prevent mutagenesis from purine base deamination within the DNA. On the other hand, the RdgB nucleotide hydrolase in *Escherichia coli* is proposed to prevent hypoxanthine and xanthine incorporation into DNA by intercepting the noncanonical DNA precursors dITP and dXTP. Because many base analogs are mutagenic when incorporated into DNA, it is intuitive to think of RdgB as acting to prevent similar mutagenesis from deaminated purines in the DNA precursor pools. To test this idea, we used a set of Claire Cupples' strains to detect changes in spontaneous mutagenesis spectra, as well as in nitrous acid-induced mutagenesis spectra, in wild-type cells and in *rdgB* single, *nfi* single, and *rdgB nfi* double mutants. We found neither a significant increase in spontaneous mutagenesis in *rdgB* and *nfi* single mutants or the double mutant nor any changes in nitrous acid-induced mutagenesis for *rdgB* mutant strains. We conclude that incorporation of deaminated purines into DNA is nonmutagenic.

Three of the four DNA bases—cytosine, adenine, and guanine—have amino groups. Deamination of these three bases (Fig. 1) generates the base analogs uracil, hypoxanthine, and xanthine, which have pairing specificities different from the original bases. Therefore, the cell employs excision repair to prevent mutagenesis due to spontaneous DNA base deamination. For example, in *Escherichia coli*, uracil-DNA glycosylase encoded by the *ung* gene initiates excision of uracils from DNA (12, 30). The *ung* mutants experience a 30-fold increase in GC→AT transitions (13), owing to the deamination of cytosine to uracil in the DNA (31). Deamination of adenine to hypoxanthine in the DNA (24) is also known to cause mutations: hypoxanthine preferentially base pairs with cytosine (3, 18, 35, 39, 51), and its formation in DNA, for example, as a result of nitrous acid exposure, leads to AT→GC transitions (45). Hypoxanthine removal from DNA in *E. coli* is initiated by the product of the *nfi* gene, endonuclease V (Endo V) (53, 54), and, to a much lesser extent, by the product of the *alkA* gene, 3-methyladenine–DNA glycosylase (43, 44). Although *nfi* mutants do not show increased spontaneous mutagenesis, they are more mutable by nitrous acid (45), an agent known to promote deamination of DNA bases (55).

Bases can deaminate not only in DNA but in nucleotides as well, both chemically (32, 46) and enzymatically (56). Deamination of DNA precursors should lead to accumulation of noncanonical nucleoside triphosphates in the cell's DNA precursor pool. These noncanonical nucleotides, such as dITP, dXTP, and dUTP, can be incorporated into the newly synthesized DNA, albeit less efficiently than regular DNA precursors (3, 38, 42, 51). Noncanonical DNA precursors are actively intercepted and removed from the DNA precursor pool. For example, dUTP is hydrolyzed to dUMP by deoxyuridine triphosphatase (2, 16), encoded by the *dut* gene (19). Indirect evidence (5; B. Budke and A. Kuzminov, unpublished data) strongly suggests that dITP and dXTP are the relevant in vivo targets of the RdgB protein, which is characterized in vitro as the dITP- and XTP-pyrophosphatase of *E. coli* (dXTP has yet to be tested) (7, 8).

Interception of noncanonical DNA precursors can be an important strategy in preventing mutagenesis due to the presence of a particular base analog in the DNA. For example, while *mutM* and *mutY* mutants, deficient in base excision repair of, or opposite to, 8-oxoguanine in DNA, show increased GC→TA transversion mutagenesis (15), *mutT* mutants, deficient in the interception of the noncanonical DNA precursor 8-oxo-dGTP, show an even stronger transversion mutagenesis in the opposite direction (AT→CG) (15). In order to see whether inactivation of *rdgB* is mutagenic due to incorporation of deaminated purines into DNA, we determined the spectra of spontaneous and nitrous acid-induced mutations in *rdgB* mutants and compared them to those of wild-type cells, *nfi* single mutants, and *rdgB nfi* double mutants.

MATERIALS AND METHODS

Strains, mutations, plasmids, and growth conditions. All strains were derivatives of *E. coli* K-12. The 11 strains from Claire Cupples (CC strains) have various *lacZ* mutations, each of which reverts by a specific base pair change or frameshift, the mutant *lacZ* genes being harbored on an F′ episome in a Δ(*pro-lac*) strain (10, 11). The CC strains were maintained on M9 plus 0.2% glucose minimal plates to select for retention of the episome (37). Otherwise, strains were routinely grown in LB broth (10 g tryptone–5 g yeast extract–5 g NaCl per liter, pH 7.2) or on LB plates (15 g agar per liter of LB broth). The *rdgB61* mutation, which is a precise deletion-replacement of the *rdgB* gene with a kanamycin resistance marker (5), was introduced into the CC strains by P1 transduction. The *nfi* gene was inactivated by transducing strains with the *nfi-1* mutation, which has a chloramphenicol resistance marker inserted at position 443 of the *nfi* gene (17). The *rdgB61* and *nfi-1* mutations were confirmed by PCR. For the *rdgB* locus, primers were YggVF4 (5′-GTCCTCGCAACCGGCAATG-3′) and YggWR2 (5′-GCATCCGGCAATCAACGTC-3′), producing a 1,819-bp product with the wild-type locus and a 2,681-bp product with the *rdgB61*::*kan* allele. For the *nfi* locus, primers were NfiF1 (5′-GTGATTATGGATCTCGC-3′) and NfiR1 (5′-CGCTCCAGACGCAGATG-3′), producing a 754-bp product with the wild-type locus and a 2,624-bp product with the *nfi-1*::*cat* allele. Antibiotics were used at the following concentrations: spectinomycin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 10 μg/ml.

Endo V-recognized DNA modifications. The 22.5-kbp plasmid pK96 (28) was introduced into each CC strain by $CaCl_2$-facilitated colony transformation. pK96 plasmid DNA was isolated from rapidly growing LB broth cultures by alkaline-

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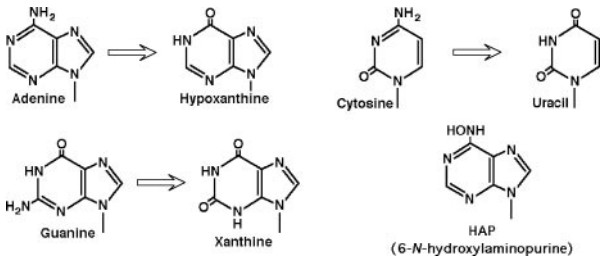

FIG. 1. Structures of the canonical DNA bases, their deaminated derivatives, and HAP.

sodium dodecyl sulfate lysis (4), and 50 ng of it was treated with 0.075 U of Endo V (Trevigen) in 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.4–50 mM NaCl–2 mM $MgCl_2$ at 37°C for 30 min, as described previously (5). The reaction was ethanol precipitated and electrophoresed on a 1.0% agarose Tris-acetate-EDTA gel for 20 h at 2.35 V/cm, after which the gel was transferred to a positively charged nylon membrane and hybridized to a pK96-specific ^{32}P-labeled probe, generated by random primer labeling. The percentage of relaxed circular plasmid DNA in the total plasmid DNA was determined by phosphorimager as the ratio of the signal from the relaxed circular band to the sum of the signals from the relaxed and supercoiled bands of pK96.

Determination of mutation frequency. Fresh colonies no more than 5 days old were grown on M9 plus glucose minimal medium and used to start 2-ml overnight LB cultures, with an initial cell density of ~10^7 CFU per ml. The cultures were incubated with shaking at 28°C for 16 to 20 h and used directly to determine the *lac* reversion frequencies for each strain except CC101, CC103, and CC106 (and their derivatives). The 2-ml overnight cultures of CC101, CC103, and CC106, the strains that gave a low yield of *lac* revertants (less than one revertant per 1.0 ml of saturated broth culture), were diluted sixfold into a total of 12 ml of LB and outgrown to saturation before everything was plated on a single plate. Each saturated culture was titered for total viable cells carrying the F′ episome by plating serial dilutions onto M9 minimal medium plates supplemented with 0.2% glucose. The concentration of *lac* revertants in each culture was then determined by plating the culture directly onto M9 minimal medium plates supplemented with 0.2% lactose, incubating the culture at 30°C for 3 days, and scoring each plate for *lac* revertants by counting uniformly large colonies. When more than 200 μl of cell culture was to be plated, the cells were collected by centrifugation and resuspended in the residual medium and then plated on the minimal medium plus lactose. In separate experiments (not shown), we made sure that the number of Lac^+ revertants per single plate increased linearly with the increasing volume of the saturated culture plated, up to at least 12 ml. The volumes of culture plated to yield 10 to 300 colonies per plate were 50 μl for CC107, CC108, and CC109; 100 μl for CC111; 1 ml for CC110; 1.5 ml for CC102, CC104, and CC105; and 12 ml for CC101, CC103, and CC106.

Nitrous acid mutagenesis. In a procedure modified from reference 17, overnight LB cultures of the strains to be mutagenized were pelleted from the volumes listed above (except for strains CC107, CC108, and CC109, which were pelleted from 1.0 ml), resuspended in 1.0 ml of 0.1 M sodium acetate buffer, pH 4.6, and titered on M9 plus glucose minimal medium. Freshly made 0.4 M $NaNO_2$ in 0.1 M acetate buffer, pH 4.6, was added to each cell suspension to yield a final concentration of 40 mM $NaNO_2$, and the suspensions were incubated at 22°C for 10 min, after which the cells were collected by a 1-min centrifugation in a microcentrifuge, resuspended in 1.0 ml of M9 salts (which effectively stopped the treatment), titered on M9 glucose minimal agar for survivors, and plated directly (100 μl for strains CC107, CC108, and CC109; 1.0 ml for all other CC strains) on M9 lactose minimal agar for *lac* revertants. Between 10 and 50% of the cells survived the treatment.

UV mutagenesis. Overnight LB cultures of strains to be mutagenized were subcultured 1:100 into fresh LB and grown to mid-logarithmic phase (optical density at 600 nm, 0.3), and 1.0 ml of the cell culture was pelleted and resuspended in 1.0 ml of 0.1 M $MgSO_4$ with 0.1% Triton X-100 to facilitate the spreading of a ~0.2-mm-thick layer of cell suspension over the rimmed top of a 9-cm plastic petri plate cover. Each culture thus spread was administered a precise dose of UV in a UV cross-linker sufficient to kill 97% of the cells in the suspension; 250 μl of the UV-irradiated suspensions were used to inoculate 5-ml LB cultures, which were shaken at 28°C overnight and then processed as described above for spontaneous mutagenesis.

MNNG mutagenesis. The *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis protocol was essentially as described previously (37). The CC cultures to be mutagenized were administered a dose of MNNG sufficient to kill 50 to 90% of the cells. Log-phase cultures were washed twice in sodium citrate buffer, pH 5.5, incubated with 150 μg/ml MNNG for 15 to 30 min, washed twice in 1× M9 minimal salts, and titered for survivors. Mutagenized cultures were outgrown 1:20 into LB overnight cultures and processed for *lac* revertants the next day.

HAP mutagenesis. 6-Hydroxylaminopurine (HAP) (MP Biomedicals) was dissolved in 100% dimethyl sulfoxide (DMSO) on a 100°C heating block at 10 mg/ml, aliquoted into microcentrifuge tubes, and stored frozen at −20°C. HAP mutagenesis was carried out by diluting saturated 2-ml overnight cultures of the wild-type and *rdgB* mutant CC strains to a 10^{-5} dilution and using 5 μl (approximately 500 cells) to inoculate a 500-μl LB culture containing either 1% DMSO and the indicated concentration of HAP or 1% DMSO alone. The cultures were grown overnight at 30°C to saturation and then processed for Lac^+ revertants according to the standard protocol; 200 μl of the overnight cultures were plated onto M9 plus 0.2% lactose minimal medium.

RESULTS

CC strains can detect increased mutagenesis. Our goal was to examine the mutagenic potential and spectra of the *rdgB61*, *nfi-1*, and *rdgB61 nfi-1* mutants of *E. coli* by using the set of CC strains (10, 11). Each of these 11 Lac^- strains detects a specific base substitution or frameshift mutation by reverting to the Lac^+ phenotype. We started by increasing the plating volumes in order to reliably detect the very rare GC→CG transversions (CC103) and AT→GC transitions (CC106) (11), which in our hands occurred about once per 10^{10} cells. We then confirmed that all of the strains of the set can detect increased mutagenesis when treated with DNA-modifying agents or combined with certain mutants.

All 11 CC strains except CC101, CC103, and CC106 responded to various extents to the nitrous acid-induced mutagenesis (Fig. 2). CC103 and CC106 did not respond to nitrous acid treatment (Fig. 2) but convincingly responded to MNNG (Fig. 3), in agreement with the literature (10, 11). We also tested CC101, CC103, CC105, and CC106 for mutagenesis with UV, but only CC105 showed a weak response (not shown). The only strain that failed to respond to any of these treatments, CC101 (AT→CG transversions), also failed to show increased mutagenesis when treated with 8-oxoguanine, hydrogen peroxide, or menadione. However, CC101 responded with more than a 10,000-fold increase in reversion to the introduction of a *mutT* mutation (Ella Rotman, personal communication). All of these tests confirm the original reports that (i) Lac^- strains of the CC set revert to Lac^+ with widely varying frequencies and (ii) in all strains, the reversion frequencies can be increased by specific DNA-modifying treatments (10, 11) or by specific mutations (52).

Spontaneous mutagenesis spectra of *rdgB* and *nfi* mutants. Next, we introduced *rdgB61* and *nfi-1* mutations into each of the 11 CC strains by P1 transduction and confirmed the mutants by PCR. Earlier we had shown that DNA of *rdgB* mutants (in an AB1157 background) accumulates modifications recognized by Endo V in vitro, but there are no such modifications in the DNA isolated from $rdgB^+$ strains independently of their *nfi* gene status (5). To make sure that deaminated purines were still incorporated into the DNA of *rdgB* mutants in the CC background, we measured the density of Endo V-recognized DNA modifications, with the help of a large plasmid (5).

The 22.5-kbp pK96 plasmid was isolated from the original 11

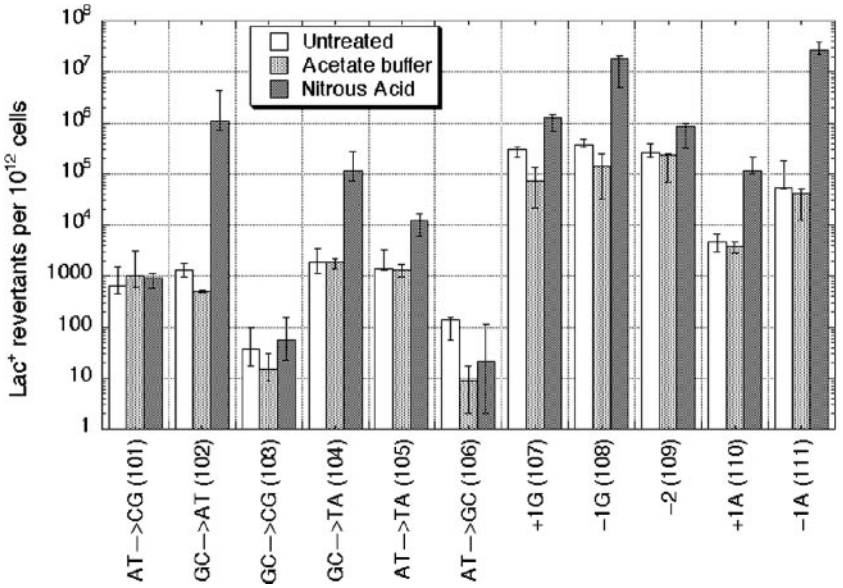

FIG. 2. Confirmation of the ability to detect increased mutagenesis in some of the CC strains after treatment with nitrous acid (in acetate buffer). The data are medians of six to nine experiments ± first and third quartiles.

CC strains, as well as from all their *rdgB* and *nfi* mutant derivatives, and treated in vitro with Endo V. The relative amount of the relaxed circular plasmid versus supercoiled plasmid was then determined by Southern hybridization. In all strains, the average level of relaxed circular pK96 in untreated samples was between 12.5% and 22%; the remainder was supercoiled plasmid (Fig. 4). When pK96 from the original CC strains, as well as from their *nfi* mutant derivatives, was treated with Endo V in vitro, there was no increase in the fraction of relaxed circular species compared to the untreated pK96 (Fig. 4). In contrast, when plasmid DNA isolated from the *rdgB* or *rdgB nfi* mutant derivatives of CC strains was treated with Endo V in vitro, the fraction of relaxed circular DNA was increased on average to 36 to 49%, suggesting that DNA of *rdgB* mutants does accumulate the expected density of Endo V-recognized DNA modifications (calculated earlier at 1 per

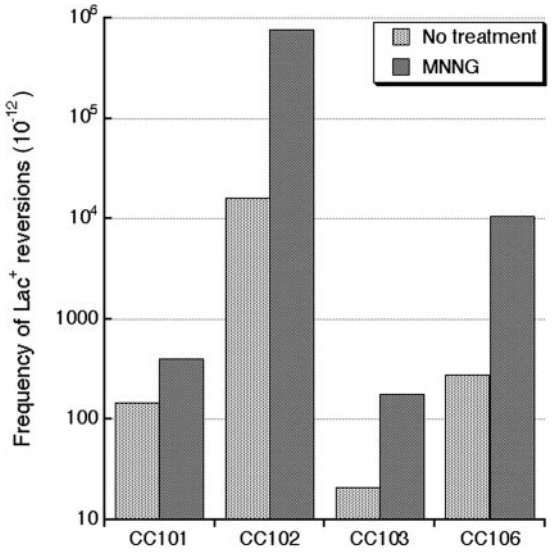

FIG. 3. Confirmation of the ability to detect increased mutagenesis in selected CC strains with MNNG. The data are averages of two experiments run on different days.

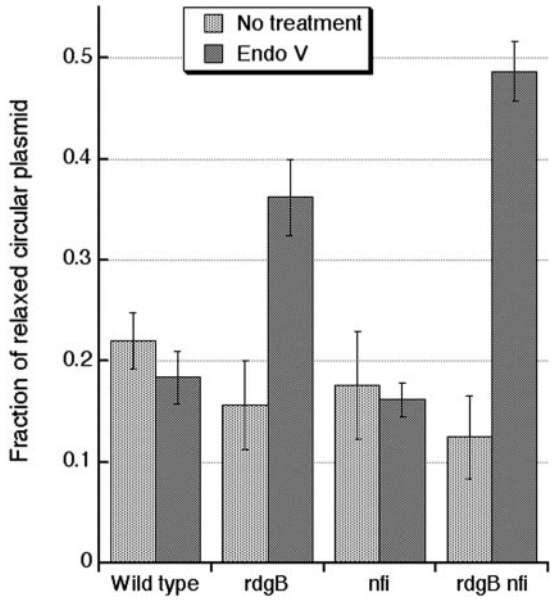

FIG. 4. Accumulation of Endo V-recognized modifications in the DNA of *rdgB* mutants. A 22.5-kbp plasmid (pK96) was isolated from the 11 CC strains, as well as from their 11 Δ*rdgB61*, 11 *nfi-1*, and 11 Δ*rdgB61 nfi-1* derivatives. The DNA was treated in vitro with Endo V, run on an agarose gel to separate supercoiled from relaxed circular species, transferred to a membrane, and hybridized with a pK96-specific probe. The fraction of relaxed circular DNA was then determined in the total plasmid preparation for every sample, and the averages for the 11 samples of the same genotype ± standard errors were plotted.

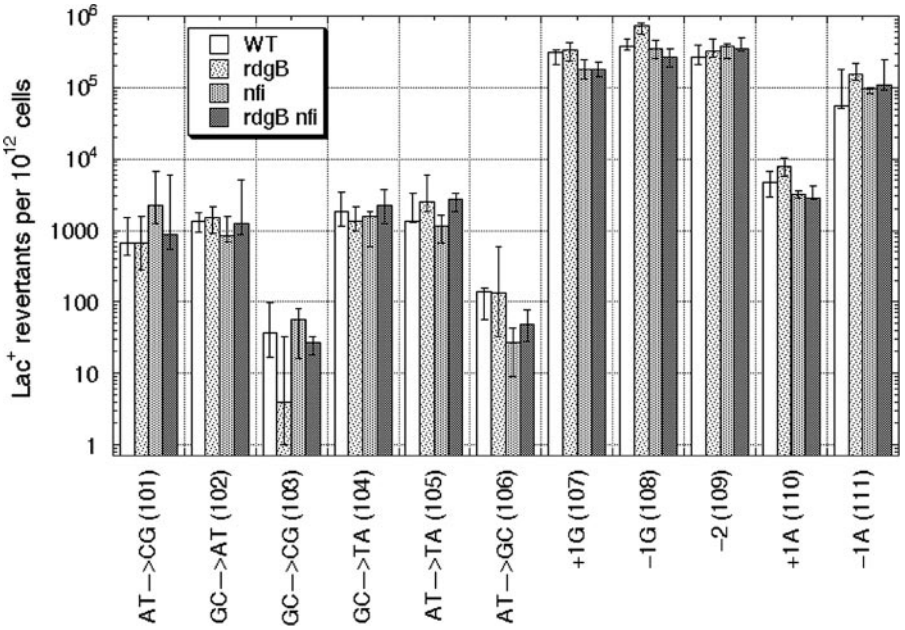

FIG. 5. Spontaneous mutagenesis in the 11 CC strains, as well as in their *rdgB*, *nfi*, and *rdgB nfi* mutant derivatives. The data are medians of eight or nine experiments ± first and third quartiles.

1.1×10^5 nucleotides [5]), presumably hypoxanthines and xanthines.

We then determined the frequencies of spontaneous reversions in the 11 CC strains, as well as in their *rdgB*, *nfi*, and *rdgB nfi* mutant derivatives. The reversion frequencies obtained from each experiment did not follow a normal distribution, owing to jackpots and experiments in which strains with low reversion frequencies did not yield any revertants. Therefore, the median was used to describe the reversion frequency of each strain. Overall, we found no substantial change in the frequency or spectrum of spontaneous mutations in either the *rdgB61* mutant or *nfi-1* mutant CC strains compared to the original $rdgB^+$ nfi^+ CC strains (Fig. 5). The double mutants also showed essentially the same spectrum (Fig. 5). We conclude that neither *rdgB* nor *nfi* defects, alone or in combination with each other, increase spontaneous mutagenesis.

Nitrous acid-induced mutagenesis. There was a possibility that the noncanonical DNA precursors intercepted by RdgB were only weakly mutagenic and that their low levels of incorporation in untreated cells (about 40 modifications per genome equivalent [5]) were not enough to cause increased mutagenesis. Acute deamination of the DNA precursors in the cell might have elevated this mutagenesis above the detection limit. On the other hand, our failure to detect mutagenic consequences of deaminated purines in the DNA precursor pools might have been due to their rapid repair in the DNA of CC strains, including *nfi-1* mutants, which were supposed to be defective in this repair. To induce acute deamination of bases in the DNA and DNA precursor pools, as well as to verify the normal kinetics of repair of deaminated DNA bases in Nfi^+ strains and the repair defect of *nfi-1* derivatives, we determined the mutation spectrum induced by a short treatment with high concentrations of nitrous acid, the only DNA-damaging agent reported to reveal the *nfi* defect (45).

Nitrous acid deaminates purine and pyrimidine bases and is known to induce GC→AT transitions due to cytosine deamination (55). In our hands, treatment with nitrous acid yielded a large increase in GC→AT transitions (2,171), GC→TA transversions (60.8), and −1 frameshifts (120.9 and 651.9) and a modest increase in AT→TA transversions (9.3) and +1 frameshifts (17.2 and 29.7) in wild-type CC strains (Fig. 6A). No significant increase in either frameshifts or base substitutions over the wild-type level was found in *rdgB61* strains mutagenized with nitrous acid, with the exception of +1 frameshifts in CC107 (Fig. 6). The *nfi* and *rdgB nfi* mutants treated with nitrous acid gave the expected (although weak in our hands) increase over the wild-type and *rdgB* single-mutant levels in GC→AT and AT→GC transitions (Fig. 6), corroborating the results of Schouten and Weiss (45). We conclude that the kinetics of deaminated base excision repair is normal in Nfi^+ derivatives of CC strains, while transition mutagenesis is elevated in *nfi* mutant derivatives of the CC set in response to nitrous acid treatment.

HAP-induced mutagenesis. Besides its "natural" substrates ITP, XTP, and dITP, RdgB is proposed to hydrolyze dHAP-TP (6), a deoxynucleoside triphosphate of the base analog HAP, which can be considered an intermediate between adenine and hypoxanthine (Fig. 1). HAP is a universal mutagen (reviewed in references 1 and 27), which is explained by the fact that in its coding capacity, HAP acts as an analog of either adenine or guanine (1, 41). Strains of the yeast *Saccharomyces cerevisiae* that are deficient in the Ham1 protein, which is an RdgB homolog, are hypermutable in the presence of HAP (27, 40). In contrast, *E. coli* controls detoxification of HAP with unknown molybdoproteins (26). In *E. coli moa* mutant strains, which are unable to synthesize the molybdenum cofactor and are thus hypersensitive to HAP, the *rdgB* defect decreases survival and increases mutagenesis after HAP treatment, sug-

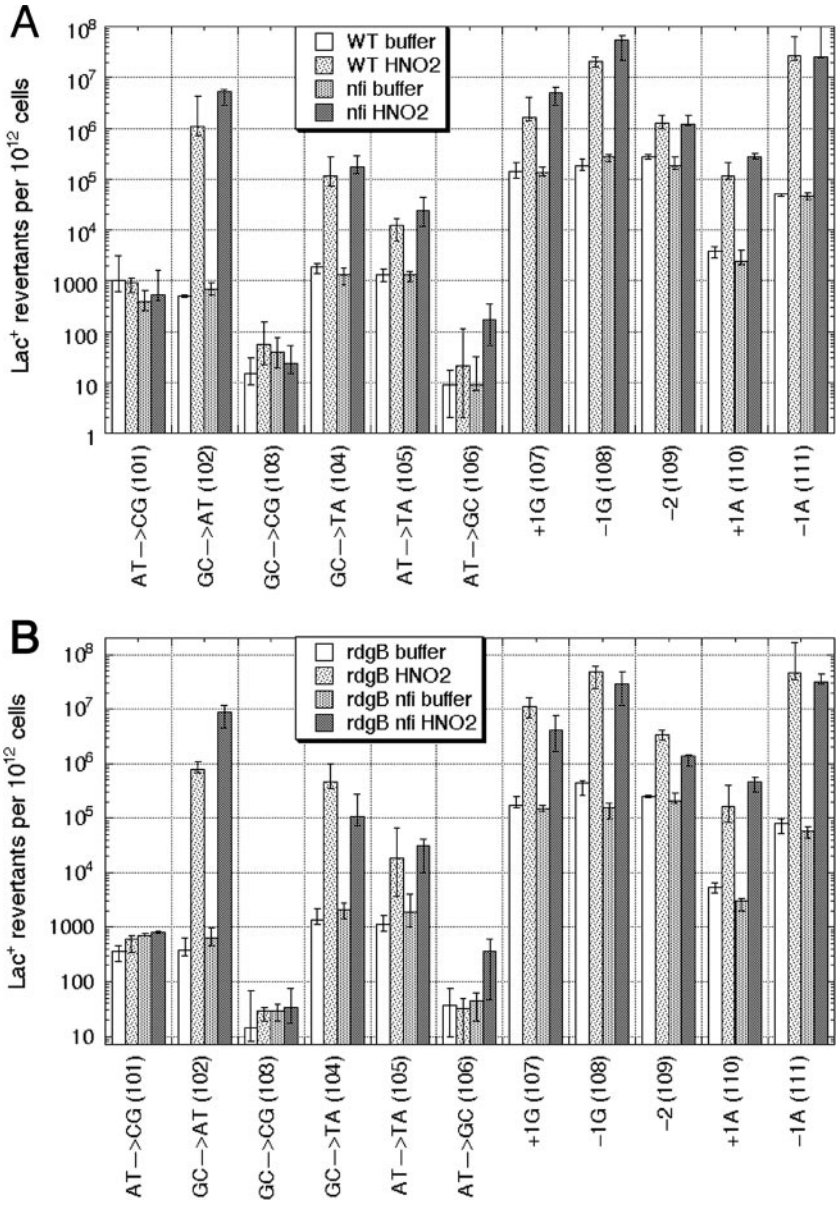

FIG. 6. Nitrous acid-induced mutagenesis. The data are medians of six experiments ± first and third quartiles. (A) Wild-type (WT) cells versus *nfi* mutants. (B) *rdgB* single mutants versus *rdgB nfi* double mutants.

gesting that RdgB of *E. coli* also intercepts the DNA precursor form of HAP, dHAP-TP (6). However, the effects of *rdgB* and *nfi* mutations on HAP mutagenesis in otherwise wild-type cells have not been evaluated.

Although we demonstrated that the *rdgB* defect has no mutagenic consequence by itself, we predicted that there would be such a consequence in cells grown in the presence of HAP. To test this prediction, we determined the magnitude of HAP-induced mutagenesis in the two transition indicator strains of the CC set (CC102, GC→AT; CC106, AT→GC) under both $RdgB^+$ and *rdgB* mutant conditions. We found that treatment with 5 μg/ml and 50 μg/ml of HAP, respectively, stimulates mutagenesis 16- to 60-fold and 45- to 95-fold in $RdgB^+$ cells (Fig. 7). Surprisingly, we did not find a significant increase in HAP-induced mutagenesis in *rdgB* mutants over the $RdgB^+$ levels (Fig. 7). At face value, this result suggests that RdgB recognizes dHAP-TP in vivo inefficiently.

DISCUSSION

Although formation of deaminated purines in DNA is mutagenic, we demonstrate that formation of deaminated purines in the DNA precursor pools and subsequent incorporation of these base analogs into DNA is not. We failed to detect any change in mutation spectra due to the *rdgB* defect, which blocks interception of dITP and dXTP in the DNA precursor pools, or due to the double *rdgB nfi* defect, which additionally prevents the incorporated deaminated purines from being excised from the DNA. Control experiments show that the strains of the CC set that we used (i) exhibit the expected

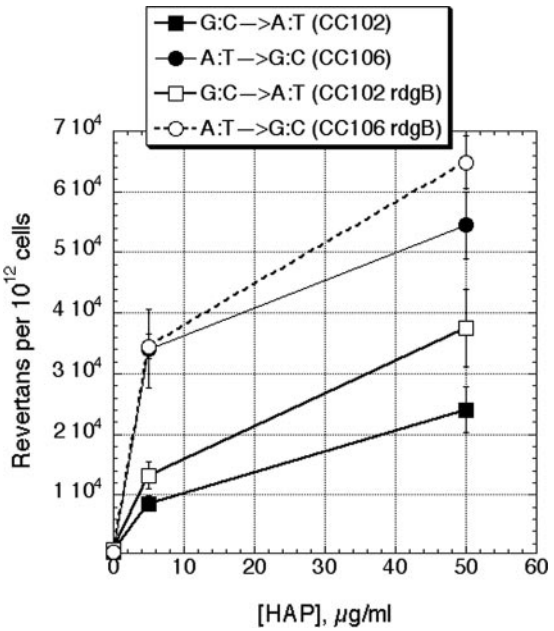

FIG. 7. HAP-induced mutagenesis. The data points are averages of four independent measurements ± standard errors.

mutagenic response to nitrous acid, confirming the normal repair kinetics of the deaminated bases in the DNA of *nfi*$^+$ cells and the lack of alternative repair pathways in the *nfi* mutant cells; (ii) accumulate Endo V-recognized DNA modifications under *rdgB* mutant conditions; and (iii) are mutable by HAP, but independently of their *rdgB* status. There should be an explanation of why the incorporated deaminated purines are so different in their mutagenic properties from purines deaminated within DNA. Since the two deaminated purines, hypoxanthine and xanthine, are quite different in their pairing characteristics while RdgB is proposed to intercept both corresponding DNA precursors, dITP and dXTP, there is likely to be more than one explanation.

It is thoroughly established that hypoxanthine, which forms in DNA due to deamination of adenine, is highly mutagenic (Fig. 8). Since hypoxanthine has a strong pairing preference for cytosine (3, 18, 39, 51), changing adenines to hypoxanthines in DNA leads to AT→GC transitions. With this fact in mind, it is intuitively apparent that incorporation of hypoxanthine in the DNA of *rdgB* mutants should be also mutagenic and that, in addition, this is probably why the cell is trying to keep hypoxanthine out of its DNA. Hypoxanthine in its DNA precursor form, dITP, is readily incorporated into DNA by isolated nuclei (38) or by purified DNA polymerases (3, 42, 51). Moreover, there is at least one report that dITP can be mildly mutagenic under PCR conditions in which one of the canonical deoxynucleoside triphosphates is limiting (49). On the other hand, neither dUTP nor dITP is mutagenic under standard replication conditions in vitro (47) or when being "transformed" at high concentrations into live *E. coli* cells (20). We would like to argue that, exactly because of its strong pairing preference for cytosine, incorporated hypoxanthine cannot be mutagenic, because it acts strictly as a guanine analog (Fig. 8). Generally speaking, only base analogs capable of alternative pairing should be mutagenic when incorporated into DNA,

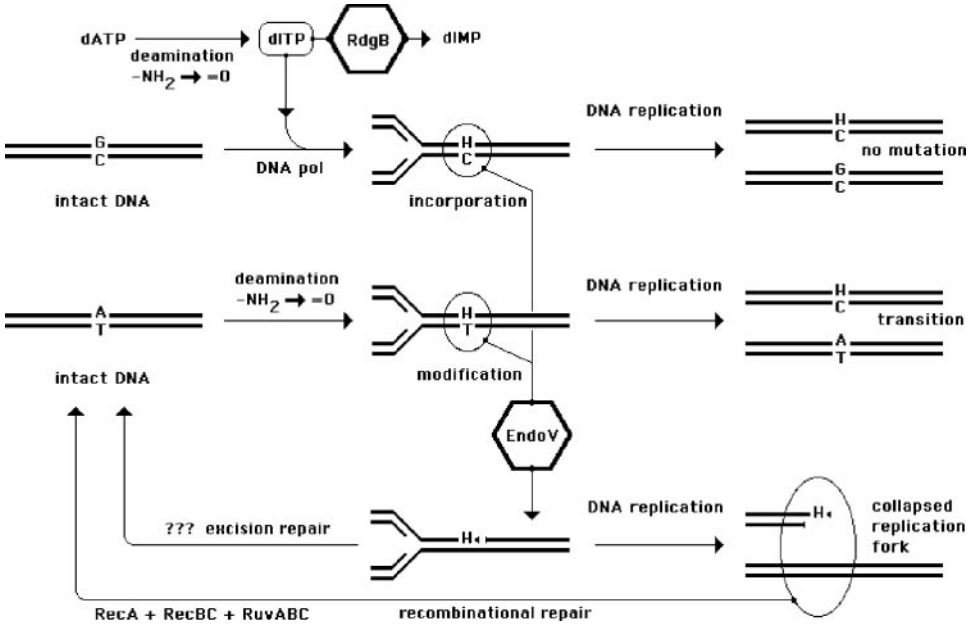

FIG. 8. Overall scheme of deaminated purine contamination and decontamination in DNA metabolism. dITP, dITP in DNA precursor pools; H, hypoxanthine in DNA; ???, lack of knowledge about excision repair of hypoxanthines downstream of Endo V nicking. Hypoxanthine in DNA may produce double-strand breaks in three ways: (i) by replication forks running into Endo V-induced nicks (shown), (ii) by direct cleavage by Endo V in single-stranded regions of the replication fork (because its hypoxanthine-specific endonuclease activity attacks both single- and double-stranded DNA [53]), and (iii) by two simultaneous Endo V nicks in the opposite strands of the same DNA duplex. We believe that the two latter mechanisms are less likely than the first one because of the very low density of Endo V-recognized DNA modifications, even in *rdgB* mutants (5).

while base analogs with strict pairing preferences cannot be mutagenic as DNA precursors. For example, uracil, which pairs only with adenine, is not mutagenic when incorporated but is highly mutagenic when formed in DNA by cytosine deamination. This peculiar point was independently raised previously (48).

Interestingly, according to this logic, the other deaminated purine, xanthine, which can pair with either thymine or cytosine (14, 23), should be highly mutagenic if incorporated into DNA. In the case of xanthine, the explanation for the lack of mutagenesis has to be entirely different. Since xanthine pairs equally poorly with both T and C, we suggest that xanthine cannot have a significant influence on mutagenesis because of the poor utilization of dXTP by DNA polymerases (3, 21, 34, 42, 50). Therefore, the combination of a strict pairing in the case of readily incorporatable hypoxanthine and an inefficient incorporation in the case of alternatively pairing xanthine leads to a somewhat counterintuitive conclusion, although fully backed by our results, that there should be no elevation of mutagenesis in *rdgB* single mutants or even in *rdgB nfi* double mutants.

As a test of this logic, we grew the two transition indicator strains in the presence of HAP, a purine base analog readily convertible into a noncanonical DNA precursor, dHAP-TP (6, 27), which incorporates into the DNA, causing transitional mutagenesis due to its approximately equal pairing with T or C (1, 41). Since it was proposed that RdgB can hydrolyze dHAP-TP in addition to dITP and dXTP (6), we expected that HAP-induced mutagenesis would be further and substantially increased in *rdgB* mutants. We indeed found a significant transitional mutagenesis in cultures grown in the presence of HAP, demonstrating the importance of intercepting the noncanonical DNA precursor dHAP-TP. However, the additional increase in mutagenesis in *rdgB* mutants exposed to HAP turned out to be statistically insignificant, suggesting that dHAP-TP is not a good substrate for RdgB in vivo.

Another test of this logic was the mutagenicity of nitrous acid, which deaminates bases in DNA as well as in DNA precursors. Nitrous acid increased the frequency of GC→AT and AT→GC transitions and GC→TA and AT→TA transversions, in addition to frameshift mutations. However, according to the above logic, nitrous acid should not induce additional mutagenesis in *rdgB* mutants, and it did not. On the other hand, the *nfi-1* mutants yielded the expected weak increase in transitional mutagenesis (45). Furthermore, we found that the *rdgB61 nfi-1* double mutants had no more nitrous acid-induced mutations than the *nfi-1* single mutants did. These results show that RdgB does not protect the cell from mutations induced by nitrous acid, further corroborating our general point that incorporation of deaminated nucleotides is nonmutagenic.

When RdgB homologs from various organisms were first implicated in sanitizing the DNA precursor pool, one of the assumptions was that these enzymes act primarily to prevent mutagenesis (7, 8, 22, 29), which is often a consequence of incorporation of base analogs into DNA, 8-oxoguanine and HAP providing good examples (27, 36). To the contrary, our findings allow us to conclude that the primary function of RdgB in *E. coli* does not include prevention of mutagenesis, despite its clear role in sanitization of the DNA precursor pools. Rather, the main function of RdgB seems to be to prevent Endo V-triggered chromosomal fragmentation (Fig. 8), as suggested by (i) the unviability of *rdgB recA*, *rdgB recBC*, and *rdgB ruv* double mutants (5, 9, 33); (ii) the significant chromosomal fragmentation in *rdgB recBC* double mutants (5, 25); and (iii) the suppression of both unviability and chromosomal fragmentation by inactivation of Endo V (5; Budke and Kuzminov, unpublished data). It remains to be tested whether the *rdgB* defect actually increases the amount of dITP and dXTP in the DNA precursor pools and whether the Endo V-recognized modifications in the DNA of *rdgB* mutants are indeed hypoxanthines and xanthines.

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